AD_____

Award Number: DAMD17-99-1-9293

TITLE: Role of ERa Splicing Variants in Regulation of AP-1 Directed Gene Transcription in Breast Cancer Cells

PRINCIPAL INVESTIGATOR: Aliccia Bollig
Richard J. Miksicek, Ph.D.

CONTRACTING ORGANIZATION: Michigan State University
East Lansing, Michigan 48824-1046

REPORT DATE: June 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2000	Annual Summary (1 Jun 99 - 31 May 00)

4. TITLE AND SUBTITLE
Role of ERa Splicing Variants in Regulation of AP-1 Directed Gene Transcription in Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-99-1-9293

6. AUTHOR(S)
Aliccia Bollig
Richard J. Miksicek, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Michigan State University
East Lansing, Michigan 48824-1046

E-MAIL:
bolligal@msu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

This report presents recently published results from biochemical analysis of estrogen receptor (ER)-alpha mRNA splicing variants (Molecular Endocrinology 14: 634-649, May 2000). Although most of the variants appear to be functionally impaired, analysis of two receptor isoforms demonstrates that they retain many of the activities of wild-type ER-alpha, including protein-protein interaction, DNA binding, ligand binding and nuclear localization. These results and additional unpublished results from reporter gene studies suggest that ER-alpha variants can positively regulate gene transcription by a non-classical mechanism. None of the variants examined activate a consensus estrogen response element (ERE). However, specific ER-alpha variants activate the promoters of some genes that are estrogen regulated even though they lack an ERE.

14. SUBJECT TERMS
Steroid Receptors, Coactivators, SRC-1, AP-1, jun, fos, IGF-1, Collagenase, Ovalbumin, TGF-beta3

15. NUMBER OF PAGES
26

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature

Mentor - Signature

6/15/00
Date

Table of Contents

Introduction

Analysis of messenger RNA (mRNA) prepared from a variety of estrogen responsive cells and tissues has established that estrogen receptor-α (ERα) mRNA is typically expressed as a mixture of transcripts (1-4). This heterogeneity results largely from a pattern of alternative mRNA splicing that gives rise to a family of correctly processed and exon-skipped ERα mRNAs. Although there is no consistent ratio of relative expression, wild-type and variant ER transcripts are always coexpressed in ER-positive tumor cell lines and normal and tumorous breast tissue. Quantitation of individual variants shows that they generally represent a minority of ER mRNA; however, as a population, splicing variants typically constitute as much as 50% of the total ER mRNA in the tissues and cell lines examined (1,2,4). While there has been extensive analysis at the RNA level of the pattern of expression and abundance of ERα splicing variants, limited information is available on their functional activity. In a recent report (*Molecular Endocrinology* 14: 634-649, May 2000) I describe results from biochemical analysis of six ERα splicing variants that arise by the deletion of one of its internal exons (ERΔE2 through ERΔE7, where the deleted exon is indicated numerically). In agreement with the functional organization of wild-type ERα (wt ERα), the loss of a particular exon results in the loss of activity ascribed to that exon (5). The deletion of an exon is also observed to disrupt activities attributed to other exons and to bestow novel function on the receptor isoform (5). Although most appear to be functionally impaired, analysis of receptor isoforms demonstrates that individual variants do retain wt ERα function with regard to protein-protein interaction, DNA binding, ligand binding and cellular localization. However, none of the variants effectively promotes gene expression from a consensus estrogen response element (ERE) (5). The traditional view of estrogen action involves hormone binding by ER to elicit a conformational change in the receptor allowing it to dimerize, bind DNA at an ERE and activate target genes (6-8). Recently a novel pathway for regulation of transcription by ER has been described that involves cooperation of the receptor with AP-1 factors (e.g., c-Jun and c-Fos) (5,9,10). An important distinction of this non-classical pathway for ER action is that functional domains within the receptor that are crucial for transactivation of an ERE appear to be dispensable for ERα activity on AP-1 directed promoters (5,9,10). This suggests a potential role for ERα splicing variants in regulation of non-classical EREs. Studies to investigate the activity of ERα variants on estrogen responsive promoters that lack a consensus ERE indicate that, depending on the promoter context, some of the splicing variants have the capacity to regulate gene expression (5, and A. Bollig and R.J. Miksicek, unpublished data). In addition to providing evidence that specific ERα splicing variants are transcriptionally active, results from studies assessing the activity of variant receptors (which can be viewed as deletion mutants of wt ERα) on noncanonical EREs may also offer insight into the mechanism of wt ERα activity in non-classical (AP-1 directed) pathways.

Summary of Research and Training Accomplishments

For the past year, the general focus of my research has been to investigate the role of ERα splicing variants in regulating gene transcription. Initial work indicated that ERα variants are ineffective at promoting reporter gene expression from an ERE; however, two of the splicing variants, ERΔE3 and ERΔE5, were observed to positively regulate gene expression from AP-1 dependent promoters that lack an ERE. In order to characterize the transactivation function of ERα splicing variants, many of my efforts have been concentrated on identifying responsive genes using a cell culture transfection system to test candidate promoters. With this same approach, I have also assessed ligand and AP-1 activator requirements for transactivation. With the evolution of my research, greater attention is being given to questions regarding the potential mechanisms of transactivation by ERα variants. In addition to further studies to determine which promoters are activated by the variants, I have completed *in vitro* protein binding studies to determine if the ERΔE3 and ERΔE5 variants retain the

ability to dimerize with wt ERα and if coactivators can interact with these variants, and thereby potentially effect their activity.

Identification of promoters activated by ERα splicing variants and assessment of AP-1 activation and hormone requirement

As reported, cDNA expression vectors containing the single exon-skipped ERα variants ERΔE2 through ERΔE7 were constructed to enable their functional characterization in a well defined cell transfection system (5). Each of these plasmids efficiently expresses a stable variant protein in Cos7 cells, as determined by western analysis, with a molecular weight consistent with its predicted translational reading frame (5). Expression vectors for wt ERα and ERα mRNA splicing variants were transiently coexpressed in HeLa cells with reporter gene constructs driven by the promoter of interest. Candidate promoters predicted to be part of a non-classical estrogen response pathway were selected based on information in the literature describing them to be regulated by AP-1 and ERα without having a consensus ERE. With this criteria I have identified four genes that are regulated by one or both of the wt ERα variants, ERΔE3 and ERΔE5. Expression of a reporter gene driven by the chicken ovalbumin promoter is induced by ERΔE5 in cells treated with phorbol 12-myristate, 13-acetate (PMA, a phorbol ester that stimulates AP-1 via a direct activation of protein kinase C) and by ERΔE3 and wt ERα with 17β-estradiol (E_2) and PMA co-treatment (ref. 5 and Appendix A, fig. 7). Overexpression of cJun enhanced wt ERα, ERΔE3 and ERΔE5 activity (ref. 5 and Appendix A, fig. 8). Estrogen regulation of the ovalbumin promoter has been mapped to a critical AP-1 enhancer motif (9).

The human collagenase promoter also harbors an AP-1 element described to direct E_2 regulated gene expression (10). A short region of the collagenase promoter (-73 to +63 relative to transcription start site) containing the critical AP-1 motif is

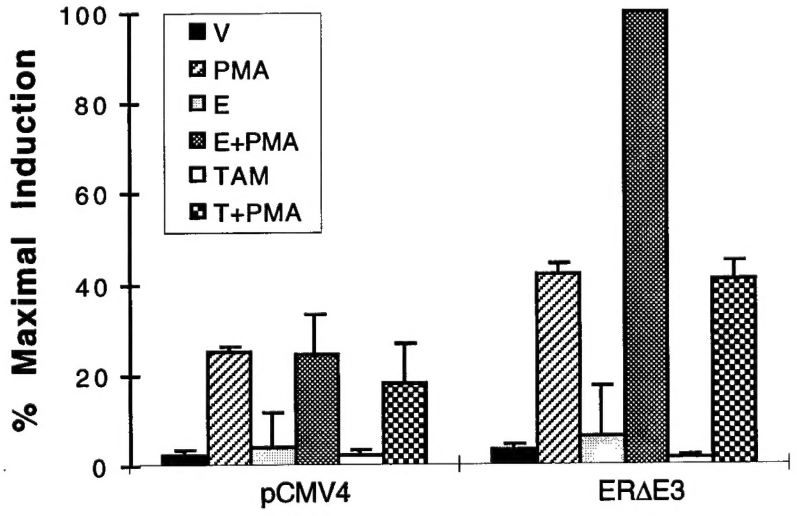

FIGURE **1.** Treatment Breakdown for the Activation of Coll73-Luc by ERΔE3
Maximal induction of the collagenase promoter by ERΔE3 requires the combined presence of PMA and E_2. The promoter demonstrates a modest response to PMA that is enhanced with cotransfected ERΔE3. Tamoxifen treatment does not support transactivation of the reporter gene. Values are expressed as a percentage of maximum induction (ERΔE3, E+PMA). *Error bars* represent the standard error of the mean of three independent experiments.

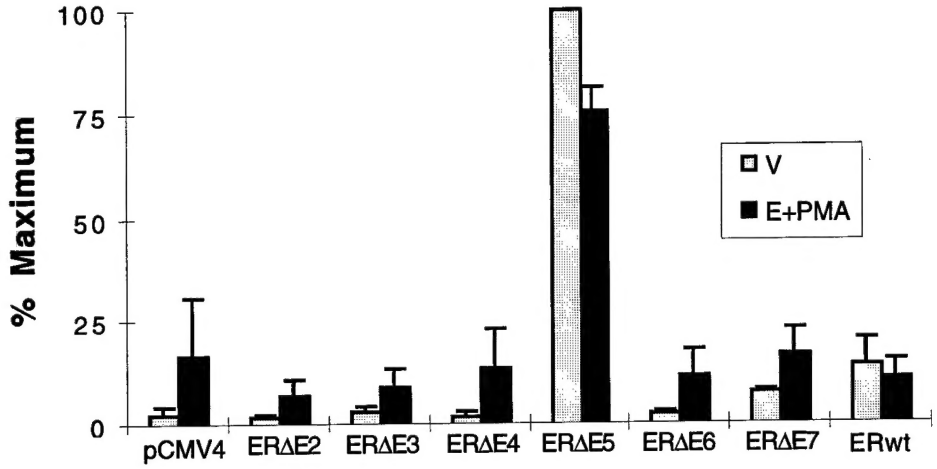

FIGURE **2.** Cotransfection of wt ERα and ERα Splicing Variants with IGF-1(1630)Luc
A luciferase reporter gene construct driven by a region of the human IGF-1 promoter (-1630 relative to transcription start site) is transactivated with cotransfection of ERΔE5. ERΔE5 activity is not enhanced with PMA treatment. However, the promoter is modestly responsive to PMA in the absence of co-transfected receptor (pCMV4). Values are expressed as a percentage of maximum induction (ERΔE5, vehicle). *Error bars* represent the standard error of the mean of 3 independent experiments.

activated by ERΔE5 and E_2-liganded ERΔE3 in the presence of PMA (see original proposal). In contrast to an earlier report from another laboratory (10), in our hands wt ERα demonstrates no activity on this promoter. Further analysis indicates that ERΔE3 activation of the collagenase promoter requires PMA and E_2 co-treatment (fig. 1). Results from reporter gene experiments using these promoters provide compelling evidence that two of the ERα mRNA splicing variants, specifically ERΔE3 and ERΔE5, can exert positive effects on gene transcription. Furthermore, although E_2 regulation for both the collagenase and the ovalbumin promoter has been mapped to an AP-1 motif, the difference in the relative strength of activation by the various receptor isoforms suggests that the context of the AP-1 element, (i.e., the flanking sequence) may confer a difference in the mechanism and pattern of regulation, as well as the structural requirements for ERα.

Another promoter that has attracted my attention is the human IGF-1 promoter. Reporter gene expression from the human IGF-1 promoter (IGF-1(1630)Luc) is induced by PMA treatment (fig. 2). The promoter does not respond to wt ERα and is constitutively induced by ERΔE5 (fig. 2). It is unclear where ERΔE5 regulation is directed in the sequence of the IGF-1 promoter, since it lacks an ERE or consensus AP-1 element (11). Efforts to transfer ERΔE5 responsiveness onto a heterologous promoter have been unsuccessful, showing that this effect requires the context of the promoter. In a further attempt to localize the ERΔE5 response element, studies are planned to test ERΔE5 activation of truncated and short deletions of the promoter.

The TGFβ3 gene is another potential target for non-classical transcriptional regulation by ERα and/or ERα splicing variants. Notably, the TGFβ3 promoter does not have an ERE, yet TGFβ3 gene expression is reportedly regulated by ER (12). A related report describes the presence of an unnusual anti-estrogen (raloxifene) response element in this promoter that is active in MG63 osteosacoma cells (13). In preliminary cotransfection experiments, PMA treatment induced E_2-bound wt ERα and ERΔE3 transactivation of the TGFβ3 promoter (data not shown). For this reason, I plan to includethe TGFβ3 reporter in future studies.

Dimerization and Coactivator Binding Properties of ERΔE3 and ERΔE5

None of the ERα mRNA splicing variants effectively activate an ERE, however, ERΔE3 and ERΔE5 are observed to influence ERE-directed transcription by disrupting wt ERα transactivation when they are coexpressed in reporter gene experiments (5,14,15). Results from *in vitro* binding assays suggest that a direct interaction between the variants and factors responsible for ERE-directed transcription might explain the inhibitory effects of ERΔE3 and ERΔE5 on wt ERα activity. Specifically, I tested for a direct interaction of ERΔE3 and ERΔE5 with wt ERα and steroid receptor coactivator-1e (SRC-1e). The C-terminus of wt ERα and fragments of the SRC-1e protein were expressed as fusion proteins with glutathione S-transferase (GST) and attached to glutathione-Sepharose beads. Binding assays with GST fused to the C-terminus of wt ERα and ^{35}S-methionine labeled *in vitro* translated receptor demonstrate that ligand-bound wt ERα and ERΔE3, but not ERΔE5, dimerize with the ligand-binding domain of ERα in solution (ref. 5 and Appendix A, fig. 6A). In experiments with GST-SRC-1e fragments, both ERΔE3 and ERΔE5 were observed to bind to regions of the coactivator that also bind wt ERα (ref. 5 and Appendix A, fig. 6B).

Training and Academic Progress

In addition to planning and executing the experiments described above, my training activities include attending a weekly Molecular Biology Journal Discussion Group in which participants present and discuss recent journal articles with relevance to their research. I have also presented my data and findings to the Michigan State University department of Physiology during an annual Research Forum. I recently addressed a meeting of my Research Advisory Committee where I presented data and conclusions as well as leading a discussion about my preliminary findings and explained my progress, and the future experiments I am planning. This year I also completed work on a manuscript that was published in the journal Molecular Endocrinology (ref. 5 and Appendix A). Prior to receiving the U.S. Army BCRP Fellowship in June 1999, I successfully completed my comprehensive examinations and defended my thesis proposal and was named a candidate for Ph.D. in the Michigan State University Department of Physiology. Presently I am in my fifth year of graduate training and am in good academic standing.

Research Accomplishments

- Analyzed the biochemical behavior of ERα splicing variants
- Completed protein interaction studies between variants and the nuclear receptor coactivator, SRC-1e
- Initiated transfection experiments to assess the effect of ER variants on gene expression from the chicken ovalbumin, human collagenase, IGF-1 and TGFβ3 promoters.

Reportable Outcomes

- Publications:

  Bollig A, Miksicek RJ 2000 An estrogen receptor-a splicing variant mediates both positive and negative effects on gene transcription. Mol Endocrinol 14:634-649

- Presentation of data and findings at a Michigan State University Department of Physiology Research Forum (January, 2000)
- Conducted a review of the progress of my research and graduate education at an annual Research Advisory Committee meeting (May, 2000)

Conclusions

- ERΔE3 dimerizes with wt ERα
- Similar to wt ERα, ERΔE3 and ERΔE5 bind SRC-1e fragments
- c-Jun cooperates with ERΔE3 and wt ERα to activate the chicken ovalbumin promoter
- ERΔE5 constituatively activates the human IGF-1 promoter. The IGF-1 promoter is not induced by wt ERα in parallel reporter gene studies.
- Activation of a hormone responsive human collagense promoter fragment by ERΔE3 in transfected cells requires dual AP-1 activation by PMA and E_2 treatment.

References

1. Castles CG, Klotz DM, Fuqua SAW, Hill SM 1995 Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. Br J Cancer 71:974-980
2. Gotteland M, Desauty G, Delarue JC, Liu L and May E 1995 Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. Mol Cell Endocrinol 112:1-13
3. Miksicek RJ, Lei Y, Wang Y 1993 Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. Breast Cancer Res Treat 26:163-179
4. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH 1997 Oestrogen receptor variants and mutations in human breast cancer. Ann Med 29:221-234
5. Bollig A, Miksicek RJ 2000 An estrogen receptor-a splicing variant mediates both positive and negative effects on gene transcription. Mol Endocrinol 14:634-649
6. Beato M, Sanchez-Pacheco A 1996 Interaction of steroid hormone receptors with the transcription initiation complex. Endocr Rev 17:587-609
7. Kumar V, Chambon P 1988 The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156
8. Tsai M-J, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451-486
9. Gaub MP, Bellard M, Scheuer I, Chambon P and Sassone-Corsi P 1990 Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. Cell 63:1267-1276
10. Uht RM, Anderson CM, Webb P and Kushner PJ 1997 Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. Endocrinology 138:2900-2908
11. Kim S-W, Lajara R, Rotwein P 1991 Structure and function of a human insulin-like growth factor-1 gene promoter. Mol Endocrinol 5:1964-1972
12. Yang NN, Bryant HU, Hardikar S, Sato M, Galvin RJS, Glasebrook AL, Termine JD 1996 Estrogen and raloxifene stimulate transforming growth factor-b3 gene expression in rat bone: a potential mechanism for estrogen- or raloxifene-mediated bone maintenance. Endocrinology 137:2075-2084
13. Yang NA, Venugopalan M, Haidikar S and Glasebrook A 1996 Identification of an estrogen response element activated by metabolites of 17β-estradiol and raloxifene. Science 273:1222-1225.
14. Ohlsson H, Lykkesfeldt AE, Madsen MW, Briand P 1998 The estrogen receptor variant lacking exon 5 has dominant negative activity in the human breast epithelial cell line HMT-3522S1. Cancer Research 58:4264-4268
15. Wang Y and Miksicek RJ 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol 5:1707-1715

An Estrogen Receptor-α Splicing Variant Mediates Both Positive and Negative Effects on Gene Transcription

Aliccia Bollig and Richard J. Miksicek

Michigan State University
Department of Physiology
East Lansing, Michigan 48824-1101

Analysis of mRNA prepared from a variety of estrogen-responsive cell lines, breast tumor specimens, and normal breast tissue have established that estrogen receptor-α (ERα) mRNA is typically expressed as a mixture of transcripts. Using PCR amplification, this heterogeneity has been shown to result largely from an imprecise pattern of mRNA splicing that gives rise to a family of correctly processed and exon-skipped ERα transcripts. We have reconstructed ERα cDNAs representing the single exon-skipped variants ERΔE2 through ERΔE7 to enable their functional characterization in a well defined cell transfection system. All six of the ERα splicing variants support the efficient expression of stable proteins in Cos7 cells, and each shows a characteristic pattern of subcellular distribution. Each of the variants displays a dramatic reduction in DNA-binding activity with a consensus estrogen response element (ERE) in an *in vitro* gel mobility shift assay. While this DNA-binding defect appears to be complete for ERΔE2, ERΔE3, ERΔE4, and ERΔE6, weak DNA binding is observed for ERΔE5 and ERΔE7. Scatchard analysis of hormone binding demonstrates that among the variants, only ERΔE3 binds 17β-estradiol (E_2) and does so with an affinity similar to wild-type ERα (wt ERα). Individual variants cotransfected with the pERE-TK-CAT reporter plasmid [a consensus ERE-driven chloramphenicol acetyltransferase (CAT) reporter gene that is highly responsive to E_2-liganded wt ERα] were ineffective at inducing CAT expression in ER-negative HeLa cells. Only ERΔE5 showed indications of positive transcriptional activity on the pERE-TK-CAT reporter, but this activity was limited to approximately 5% of the activity of wt ERα. When variants were expressed simultaneously with wt ERα, ERΔE3 and ERΔE5 were observed to have a dominant negative effect on wt ERα transcriptional activity. Like the wild-type receptor, both ERΔE3 and ERΔE5 interact with steroid receptor coactivator-1e (SRC-1e) *in vitro*; however, only ERΔE3 retained the ability to dimerize with wt ERα. Transcription from a region of the ovalbumin promoter, which contains an ERE half-site and an AP-1 motif, is positively regulated by liganded wt ERα and ERΔE3 in phorbol ester-treated, transiently transfected HeLa cells. In both cases, this activity was enhanced by cotransfected cJun. These observations suggest that selected ERα splicing variants are likely to exert important transcriptional effects, especially on genes that are regulated by nonconsensus EREs and subject to complex hormonal control. (Molecular Endocrinology 14: 634–649, 2000)

0888-8809/00/$3.00/0
Molecular Endocrinology 14(5): 634–649

INTRODUCTION

Binding of estrogen to the estrogen receptor (ER) elicits a change in receptor conformation that allows the receptor to bind DNA and enhance transcription from the promoters of regulated genes (1–3). ER-induced gene expression supports the proliferation and, ultimately, the differentiation of target cells (4, 5). Interference with these proliferative effects forms the basis for the chemotherapeutic actions of estrogen antagonists that are used to treat cancers of the breast and reproductive tract (6). The reported success of antiestrogens such as tamoxifen and raloxifene in preventing breast tumors emphasizes a crucial role for ER in mammary carcinogenesis (7–9).

The transcriptional effects of estrogens are mediated by two closely related receptor isoforms, ERα and the more recently described ERβ (10, 11), each of which is encoded by a separate gene. While ERβ is also being investigated for its potential role in various diseases, including cancer, this study focuses solely on the ERα isoform. Analysis of mRNA prepared from a variety of estrogen-responsive cells and tissues, including breast tumors, has established that ERα mRNA is typically expressed as a mixture of transcripts (12–15). This heterogeneity results largely from a pattern of alternative mRNA splicing that gives rise to a family of correctly pro-

cessed and exon-skipped ERα mRNAs. ERα mRNA comprises sequences from 8 coding exons and is translated to yield a protein with discrete functional domains. An N-terminal transactivation function (AF1) encoded by exon 1 and a portion of exon 2 is thought to promote gene transcription by interacting with nuclear receptor coactivators and also with proteins integral to the transcription initiation complex (1, 16, 17). Derived from exons 2 and 3 is a centrally located zinc-finger motif (commonly referred to as the DNA-binding domain or DBD) that is essential for sequence-specific DNA binding and transcriptional activation through canonical estrogen response elements (EREs) (18). Within the region encoded by exon 4 are the nuclear localization signals (NLS) and a hinge region that allows for receptor conformational flexibility (3, 19). A ligand-binding domain (LBD) confers regulatory function to the receptor and is encoded by the C-terminal exons 4 through 8 (20). This region also includes determinants for subunit dimerization and a well characterized C-terminal transactivation function (AF2), which promotes gene transcription by recruiting coactivators (1–3, 21). Like other nuclear receptors, ERα is a modular protein in that individual domains are capable of demonstrating autonomous function within receptor mutants, as well as when they are introduced into heterologous fusion proteins (1, 18). It can reasonably be assumed that the exclusion of a particular exon will predictably result in a protein lacking the function ascribed to that exon. Additionally, it is probable that the loss of a particular exon will result in unpredictable functional deficits or perhaps even bestow a novel function on the variant receptor. This study examines the function of ERα splicing variants from the vantage point of what is known about the functional organization of wt ERα. Concurrently, the process of examining splicing variants, like mutational studies, improves our understanding of wt ERα function. We report results from experiments designed to assess receptor capacity to translocate to the nucleus, bind to DNA, bind ligand, participate in protein complexes, and promote gene transcription.

Fuqua and colleagues (22, 23) have reported that ERΔE5 (which contains the AF1 domain, but lacks AF2 and the regulatory functions imparted by the LBD), is constitutively active in promoting transcription from an ERE in a heterologous yeast reporter gene assay. These authors have also described that overexpression of ERΔE5 in a stably transfected breast cancer cell line (MCF-7) supported greater proliferation compared with control cells, as well as imparting a tamoxifen-resistant phenotype (24). In the human osteosarcoma cell line U2-OS, it has recently been reported that coexpression of ERΔE5 significantly enhances ERE-directed reporter gene expression induced by wt ERα (25). The existence of a constitutively active receptor variant (such as ERΔE5) able to exert a mitogenic effect in breast tumor cells in the absence of E_2 or in the presence of tamoxifen is an appealing explanation for the acquisition of antiestrogen resistance observed in previously responsive tumors and cell lines (26, 27). However, this model is challenged by conflicting observations that ERΔE5 and closely related, genetically engineered ERα mutants do not efficiently induce transcription from an ERE reporter in transiently transfected ER-negative HeLa or CEF cells (2, 28), or promote proliferation in stably transfected MCF-7 cells (28).

Recently, a novel mechanism for mediation of an estrogen response has been reported to involve AP-1-directed regulation of transcription by ER (29–32). AP-1 describes the fos/jun family of transcription factors that play a key role in transducing the effects of growth factors to regulate cell proliferation (33, 34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements (5′-TGAG/CTCA-3′), with or without a degenerate ERE or ERE half-site (5′-GGTCA-3′ or 5′-TGACC-3′). Examples of such genes include ovalbumin, which is induced by E_2 in chicken oviduct cells (35), and the insulin-like growth factor-I (IGF-I) gene whose expression is stimulated by E_2 in the uterus of ovariectomized-hypophysectomized rats and in cultured rat osteoblast cells (36, 37). An AP-1 enhancer motif identified in the chicken IGF-I promoter is essential for E_2 and phorbol ester-stimulated gene transcription (31). Phorbol esters act directly on protein kinase C to initiate a signal transduction cascade that ultimately activates AP-1 (33). Reporter gene cotransfection studies with expression vectors for AP-1 isoforms and ERα in HeLa cells indicate that a similar mechanism regulates the human collagenase promoter (32). The minimal region of the collagenase promoter reported to be responsive to tamoxifen-liganded wt ERα, and to a variety of ERα mutants, harbors a critical AP-1 element and lacks a consensus ERE. Additionally, the activity of ERα on the collagenase promoter was enhanced with AP-1 (c-*jun* or c-*fos*) overexpression (32). Further evidence that ER regulation converges with AP-1-directed gene transcription is provided by results from protein binding assays indicating that c-*jun* is able to bind to wt ERα *in vitro* (32).

Although evidence for function of ERα variants has been elusive, reports that ERΔE5 can support weak, cell type-dependent activity (23, 25, 28), and that, when tested on an ERE, both ERΔE5 and ERΔE3 are dominant negative receptor forms in the presence of wt ERα (38, 39) indicate that it is inaccurate to label these variants as transcriptionally inert. To investigate the capacity for ERα splicing variants to regulate gene transcription, we have expanded our transcriptional focus to include the noncanonical ERE of the ovalbumin promoter in addition to the consensus vitellogenin A2 ERE. Here we present data indicating that individual variants display both similarities and differences compared with wt ERα, and that selected splicing variants (specifically ERΔE3 and ERΔE5) have the ca-

pacity to both positively and negatively regulate gene expression, depending on the promoter context.

RESULTS

Numerous variant ERα cDNAs have now been cloned and sequenced from breast tumors and established tumor cell lines (12–15). The most common variants harbor a precise deletion of one of the internal exons from the eight that contribute to the structure of the mature ERα protein, suggesting that they arise as a result of imprecise splicing of the primary ERα mRNA transcript. ERα cDNAs with sequence deletions corresponding to exons 2, 3, 4, 5, and 7 have been identified, along with a large number of more complex variants (12–15). These basic variants will be referred to as ERΔE2 through ERΔE7, where the deleted exon is indicated numerically. Although there is no consistent ratio of relative expression, wild-type and variant ER transcripts are always coexpressed in ER- positive tumor cell lines and normal and tumorous breast tissue. Quantitation of individual variants shows that they generally represent a minority of ER mRNA; however, as a population, splicing variants typically constitute as much as 50% of the total ER mRNA in the tissues and cell lines examined (Refs. 12, 13, and 15 and D. P. Ankrapp and R. J. Miksicek, unpublished observations). While there has been extensive analysis at the RNA level of the pattern of expression and abundance of ERα splicing variants, limited information is available on their functional activity. We have therefore constructed cytomegalovirus (CMV) promoter-driven ERα cDNA expression vectors representing the exon-skipped variants ERΔE2 through ERΔE7 to enable their functional characterization in a well defined cell transfection system. Our assembled pool of ERα splicing variants also includes the hypothetical receptor ERΔE6, even though this variant is not readily identified *in vivo*. Figure 1A diagrams the ERα mRNA splicing variants examined, showing the positions of deleted exons and their consequences with respect to protein structure. Deletion of exon 2, 5, 6, or 7 all cause a frame-shift mutation resulting in premature termination of translation, thereby generating a diverse class of C-terminally truncated receptor forms. Omission of either exon 3 or 4 does not disrupt the mRNA reading frame, but produces a receptor protein with an internal deletion.

Transient expression in Cos7 cells demonstrates that each of these variants translates to a stable protein able to accumulate to readily detectable levels within transfected cells (Fig. 1B). Based on immunoblot analysis with an N-terminal monoclonal antibody (Mab-17), which recognizes an epitope within exon 1 common to all of the variants (40), we observe that the mobility of the six variant proteins is consistent with their predicted molecular weights. No immunoreactivty is observed in mock transfected cells, confirming the specificity of the Mab-17 antibody.

Measurement of the DNA-Binding Activity of the ERα Splicing Variants

Efficient DNA binding by ERα requires the cooperation of several functional elements within this protein, including the centrally located DBD and a ligand-inducible subunit dimerization motif located near the C-terminal end of the LBD (2, 18). It is also possible that additional subunit contacts occur elsewhere in the protein. Because all of the ERα splicing variants sustained deletions within various regions of this protein, it was of interest to systematically assess the DNA-binding ability of each variant. For this purpose, gel mobility shift assays were performed using extracts prepared from E_2-treated, transiently transfected Cos7 cells. Extracts were incubated with a ^{32}P-labeled oligonucleotide containing a consensus ERE (AGGTCACAGTGACCT) from the *Xenopus* vitellogenin A2 promoter. As expected, variants that harbor a mutation within the DBD (ERΔE2 and ERΔE3) are completely unable to recognize the ERE (Fig. 2, lanes 5–8). Less predictably, the loss of exons contributing to the LBD also result in a strong defect in ERE recognition (Fig. 2, lanes 9–16). For ERΔE5 and ERΔE7, however, this appears to be a quantitative defect in DNA binding. The addition of the monoclonal antibody, MAb-17, to the binding reactions consistently results in the recovery of weak DNA binding by ERΔE5 (Fig. 2, lane 12). Presumably, the role of the bivalent antibody is to stabilize the interaction of receptor subunits with their palindromic binding site, mimicking the function of the missing dimerization motif present within the C terminus of the LBD. These results suggest the possible existence of cell-specific constituents that perform the same function *in vivo* and may account for the variable activity of ERΔE5 and related constructs in different cell types (2, 25, 28). We have also observed the formation of a complex between ERΔE7 and labeled ERE probe (Fig. 2, lane 16, and data not shown). Overall, however, the relative weakness of DNA-binding observed in these studies raises serious questions about the extent to which any of the variants, including ERΔE5 and ERΔE7, are able to recognize and bind to a consensus ERE, *in vivo*. Furthermore, that ERΔE7 binds an ERE *in vitro* has little transcriptional relevance in light of the observation that ERΔE7 is not translocated to the nucleus when expressed in Cos 7 cells (see below).

ERΔE3, Like wt ERα, Binds Ligand

To test the ability of the ERα mRNA splicing variants to bind hormone, we performed a saturation binding assay on whole-cell extracts from Cos7 cells transiently transfected with wt ERα or the ERα variants. Only wt ERα and ERΔE3 were able to bind ^{3}H-labeled E_2, whereas all of the remaining variants demonstrated no specific ligand binding (Fig. 3A). The individual deletion of exons 2, 4, 5, 6, and 7 effectively eliminates all, or a significant portion, of the LBD (see Fig. 1A), consistent with their loss of hormone binding. We confirmed these results using an *in vivo* ligand-binding

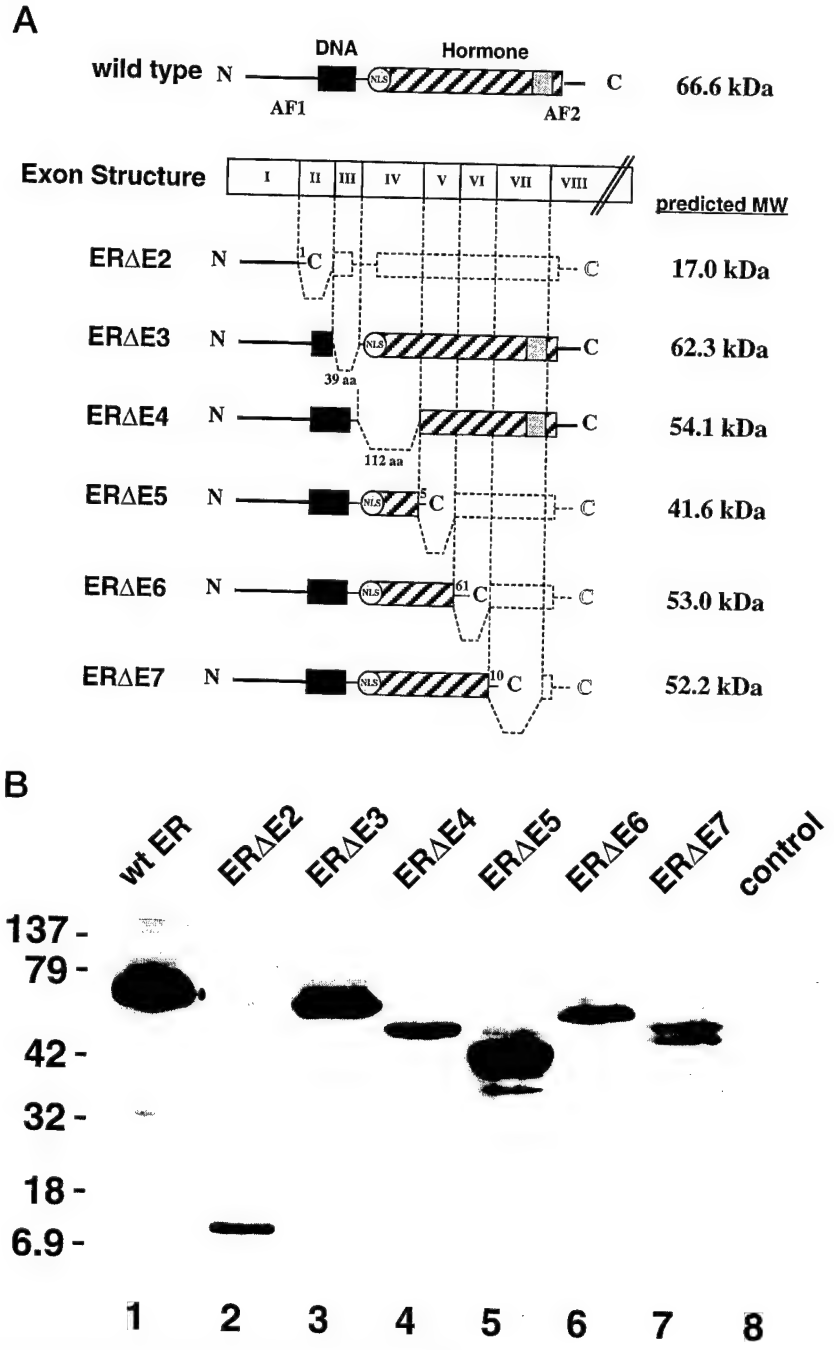

Fig. 1. Comparison of ERα mRNA Splicing Variants and wt ERα Structure

Panel A depicts the various functional domains of the receptor and the exon sequences from which they are derived. The variants are referred to by deleted exon. The size and mol wt of each variant are predicted from the translational reading frame of the sequenced cDNA clones. *Dashed lines* indicate regions of the major open reading frame of the full-length ERα protein that are missing from each variant. The nuclear localization signal is *circled*. The regions encompassing the DNA- and hormone-binding domains are marked by *darkened and hatched boxes*, respectively, where they are expressed. The AF-1 and AF-2 domains are indicated where they reside within the N and C termini, respectively, of the wild-type receptor. B, Immunoblot analysis indicates that the mol wt of each variant is consistent with its predicted translational reading frame. Samples containing 20 μg of protein from extracts of transfected Cos 7 cells were analyzed on a 10% SDS-polyacrylamide gel probed with the ERα-specific antibody, Mab-17. The figure is representative of three independent transfection experiments.

assay in which the binding of a fluorescent estrogen analog was visualized in cells cultured on cover slips. Cos7 cells transiently expressing the individual variants or wt receptor were treated with the fluorescent ligand, nitrile tetrahydrochrysene (nitrile THC) (41). Only those cultures transfected with wt ERα or ERΔE3

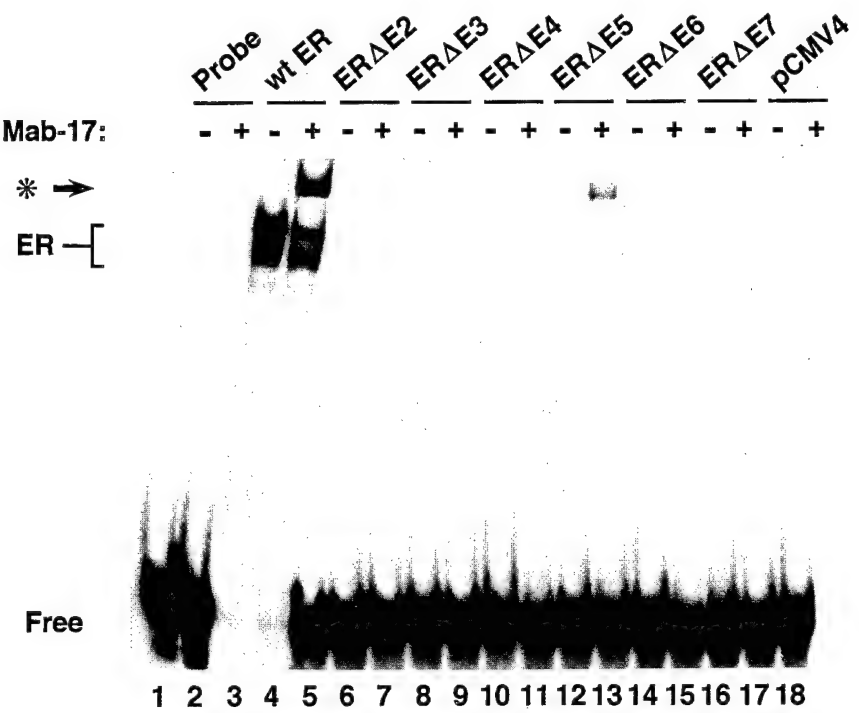

Fig. 2. Gel Mobility Shift Assay to Assess the DNA-Binding Activity of ERα mRNA Splicing Variants

A gel mobility shift assay of lysates from E_2-treated, transiently transfected Cos7 cells confirms that wt ERα binds efficiently to a ^{32}P-labeled consensus ERE. Confirmation that the indicated band represents an authentic ERα/DNA complex is provided by the ability of an ERα-specific monoclonal antibody (Mab-17) to supershift this complex (compare lane 4 with lane 3). In contrast, all of the splicing variants display strong defects in DNA binding. In this assay, which is representative of three equivalent and independent experiments, weak binding of ERΔE5 and ERΔE7 to the ERE can only be observed when their respective DNA complexes are stabilized by the addition of the ERα-specific antibody (lanes 12 and 16). Position of the antibody-supershifted complex is indicated by an *asterisk*.

were observed to stain with this ligand. In both cases, staining was localized tightly within the nucleus. This suggests that among the variants examined, wt ERα and ERΔE3 exclusively bind ligand and in both cases, ligand-bound receptors are translocated normally to the nucleus of expressing cells (Fig. 3B). With Scatchard analysis we compared the affinity of ERΔE3 and wt ERα for ^{3}H-labeled E_2. The measured dissociation constants were 0.66 nM for wt ERα and 0.79 nM for ERΔE3 (Fig. 3C).

Subcellular Localization of ERα Splicing Variants

To more carefully assess the subcellular localization of ERα splicing variants, including those that fail to bind ligand, Cos7 cells were transiently transfected with expression vectors encoding wt ERα or individual variants. These receptors were detected in transfected cells by indirect immunofluorescence staining (using the MAb-17 monoclonal antibody) and confocal microscopy. Similar to wt ERα, ERΔE3 and ERΔE5 localize to the nuclei of transfected cells, although ERΔE5 showed perinuclear as well as nuclear staining (Fig. 4). These results are consistent with the fact that both ERΔE3 and ERΔE5 retain a NLS immediately downstream of the DBD (3, 19). Subcellular localization studies have also been completed for the exon 2, 4, 6, and 7 deletion variants. Each of these proteins can be readily detected in transfected cells, but they all possess dramatic defects in nuclear targeting (Fig. 4). Nuclear targeting of wt ERα is governed, in large part, by a tripartite karyophilic signal present within exon 4 (19). Loss of this signal is therefore consistent with the cytoplasmic pattern of distribution of mutants such as ERΔE2 and ERΔE4, both of which lack protein corresponding to exon 4 sequences. Inappropriate presentation or folding of this signal must account for the defects in nuclear localization seen with ERΔE6 and ERΔE7, since the NLS is retained in these variants. Based on their subcellular distribution, we would predict that only ERΔE3 and ERΔE5, like wt ERα, would have the potential to exert nuclear effects, such as modulating gene transcription. Furthermore, the inability of the cytoplasmic variants ERΔE2, ERΔE4, ERΔE6, and ERΔE7 to dimerize with wt ERα predicts that their subcellular distribution will not be influenced by coexpression with the nuclear isoforms (wt ERα, ERΔE3, and ERΔE5). For ERΔE4, this was confirmed with a cotransport assay using a dimerization-competent ERα (data not

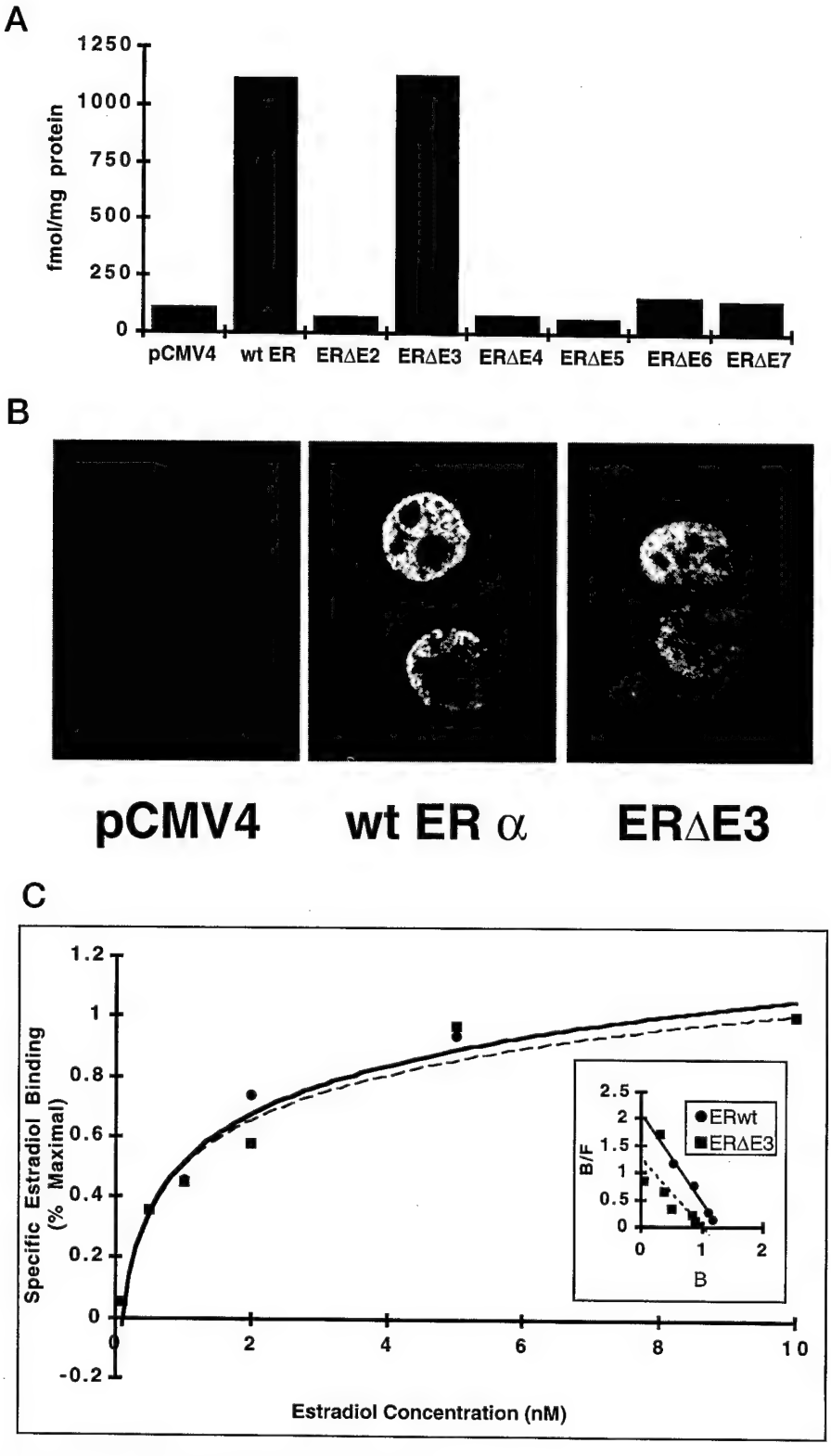

Fig. 3. Ligand Binding of ERα and ERα mRNA Splicing Variants

A, Ligand-binding capacity was assessed by measuring the specific association of 10 nM ^{3}H-17β-E_2 with wt ERα and its splicing variants expressed in Cos7 cells. Only wt ERα and the ERΔE3 variant demonstrate specific binding of E_2. B, Confirming the ligand binding results, ERΔE3 and wt ERα are the only isoforms of the receptor observed to display specific cell staining using a fluorescent estrogen analog (nitrile THC) to treat transiently transfected Cos7 cells. C, Scatchard analysis shows that wt ERα and ERΔE3 have similar affinities for 17β-estradiol.

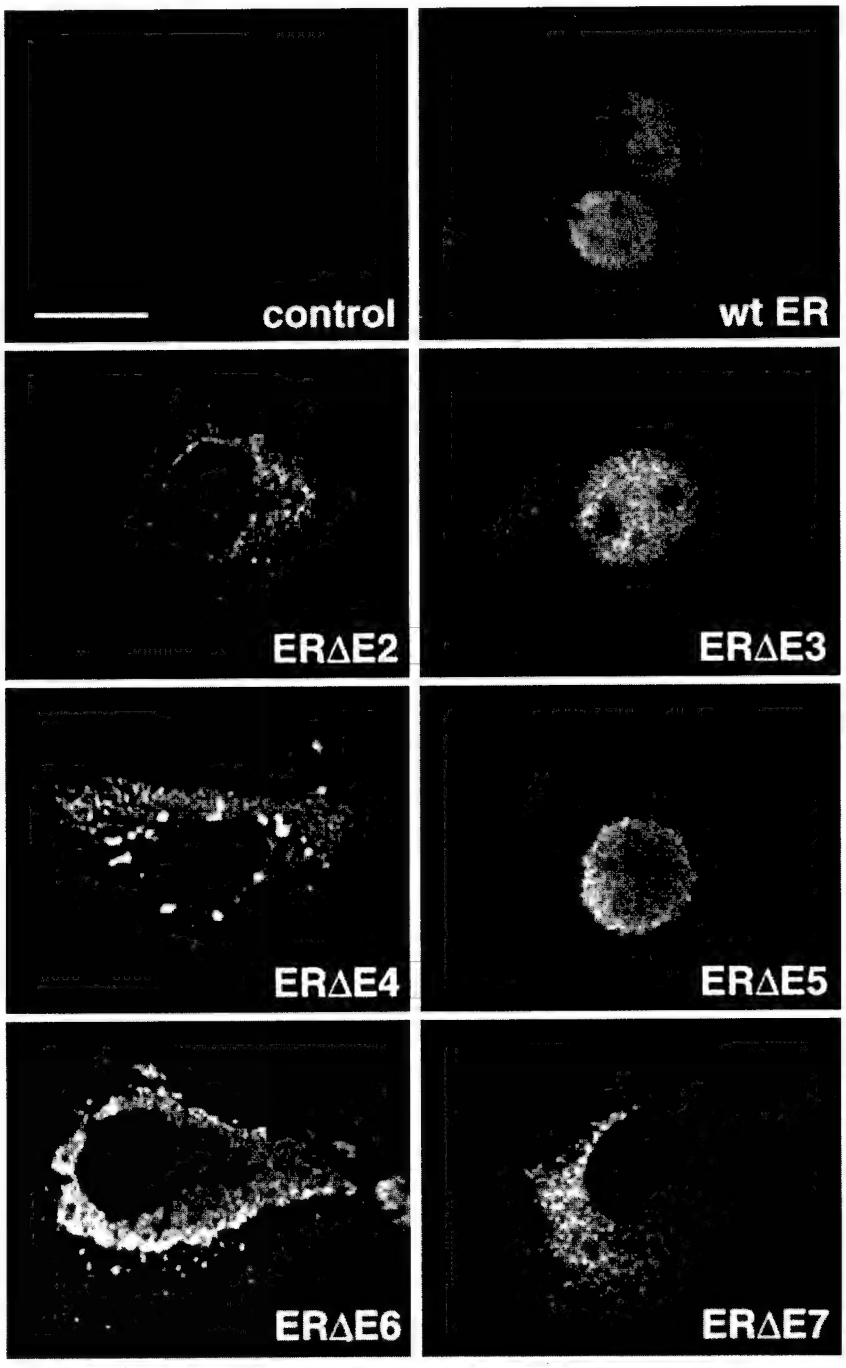

Fig. 4. Localization of ERα and ERα Splicing Variants by Confocal Microscopy

Receptor isoforms were detected by immunofluorescence staining with an ERα-specific monoclonal antibody (Mab-17) and a rhodamine-conjugated secondary antibody. The *upper left panel* shows a representative field from control cells transfected with empty expression vector (pCMV4) displaying minimal nonspecific background. Specific immunoreactivity can be observed in all other frames. Cells expressing wt ERα, ERΔE3, and ERΔE5 demonstrate strong nuclear staining in confocal sections. ERΔE2, ERΔE4, ERΔE6, and ERΔE7 are predominately localized to the cytoplasm. *Bar*, 10 μm.

shown). It is worth noting, moreover, that the existence of translationally stable, cytoplasmic splicing variants such as these may provide an explanation for cytoplasmic staining that is commonly observed during immunohistochemical analysis of breast biopsy specimens to assess ERα status.

Characterization of the Transactivation Function of ERα Splicing Variants on the Vitellogenin ERE

HeLa cell cotransfection experiments designed to assess the transcriptional activity of individually expressed ERα splicing variants have failed to demon-

strate any significant ability of variant receptors to support gene activation through an ERE, with the possible exception of the ERΔE5 variant, which is reported to display a low level of constitutive transcriptional activity on an ERE-driven reporter in some, but not all, cell types examined (23, 25, 28). In our hands none of the variants were effective transcriptional activators of an ERE-containing promoter. ERΔE5 repeatedly showed only modest constitutive activity (~5% of wt ERα induction) on an ERE-directed reporter plasmid cotransfected into HeLa cells (see Fig. 7, *inset*).

It is important to recognize that the tissues and cell lines that express these variants also express wt ERα. We have previously reported that the ERΔE3 variant acts as a dominant negative mutant when it is coexpressed with wt ERα in HeLa cells treated with E_2 (39). In the human breast epithelial cell line HMT-3522S1, ERΔE5 has also been reported to disrupt transactivation by agonist-bound wt ERα of an ERE reporter gene (38). To clarify whether this is a function unique to these variants, we completed a series of experiments to test whether the remaining exon-skipped ERα variants also support transcriptional inhibitory effects. When examined in a HeLa cell cotransfection assay in which the expression of pERE-TK-CAT was driven by E_2-bound wt ERα, a 5-fold molar excess of any of the splicing variants lacking exons 2, 4, 6, or 7 failed to inhibit the E_2-dependent induction of chloramphenicol acetyltransferase (CAT) gene expression by intact receptor (data not shown). In agreement with previously published results, the ERΔE3 and ERΔE5 variants both demonstrated a dominant inhibitory activity at all molar ratios tested (Fig. 5). With the caveat that equal amounts of plasmid DNA support similar levels of variant receptor expression (see Fig. 1B), it appears that ERΔE3 and ERΔE5 are approximately equivalent in their inhibitory activity in HeLa cells.

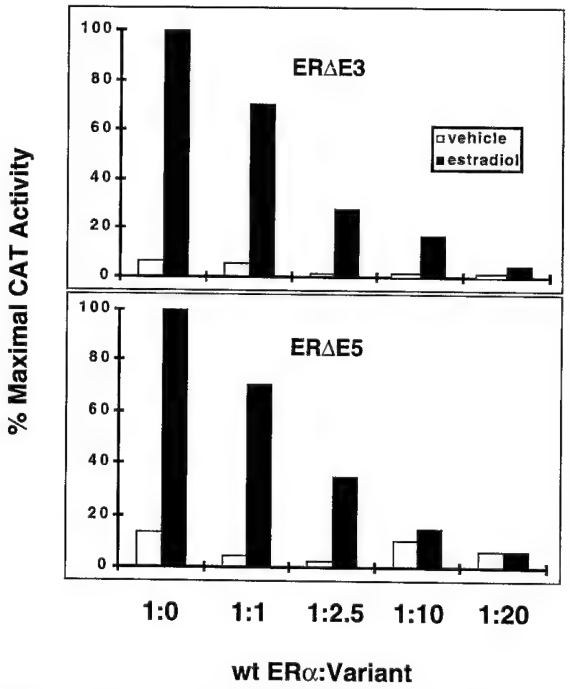

Fig. 5. ERΔE3 and ERΔE5 Inhibit Transactivation of a Consensus ERE Reporter by wt ERα in a Dose-Related Fashion

HeLa cells were cotransfected with 16 μg of pERE-TK-CAT reporter gene, 1 μg of wt ERα expression vector, and increasing amounts of expression vectors for ERΔE3 or ERΔE5 (from 0–20 μg). The ratios of wt ERα to variant expression plasmid used in each transfection are indicated. The total amount of DNA in each transfection was held constant with the addition of empty expression vector, pCMV4. Reporter gene expression was normalized by measuring CAT activity in aliquots representing 100 μg of soluble protein.

Dimerization and Coactivator Binding Properties of ERΔE3 and ERΔE5

We next questioned whether a direct interaction between the variants and factors responsible for ERE-directed transcription might explain the inhibitory effects of ERΔE3 and ERΔE5 on wt ERα activity; specifically, we tested for a direct interaction of ERΔE3 and ERΔE5 with wt ERα and steroid receptor coactivator-1e (SRC-1e). The C terminus of wt ERα and fragments of the SRC-1e protein were expressed as fusion proteins with glutathione *S*-transferase (GST) and attached to glutathione-Sepharose beads. Binding assays with GST fused to the C terminus of wt ERα (GST-AF2) and ^{35}S-methionine labeled *in vitro* translated receptor demonstrate that ligand-bound wt ERα and ERΔE3, but not ERΔE5, dimerize with the LBD of ERα in solution (Fig. 6A). In experiments with GST-SRC-1e fragments, both ERΔE3 and ERΔE5 were observed to bind to regions of the coactivator that also bind wt ERα (Fig. 6B). ERΔE3 and wt ERα bind the SRC-1e fragments comprising amino acids 570–780 and 989-1240 and do so only in the presence of E_2. In contrast, binding of ERΔE5 to the 989-1240 amino acid fragment is constitutive (Fig. 6B).

ERΔE3 Is a Positive Regulator of Gene Expression on an AP-1 Reporter

The results presented above indicate that ERα splicing variants are either inactive (ERΔE2, ERΔE4, ERΔE6, and ERΔE7) or largely inhibitory (ERΔE3 and ERΔE5) in their effect on reporter constructs that contain a consensus ERE. Recent reports suggest that wt ERα is also able to transactivate genes whose promoters do not contain an obvious ERE. In particular are promoters for the human collagenase, chicken IGF-I and ovalbumin genes that are regulated by wt ERα and contain a critical AP-1 element (29, 31, 32). Mutational analysis revealed that the DBD was not required for ERα-dependent expression of these genes. Clearly, the mechanisms of ERα transcriptional activity and DNA targeting are complicated by these reports. We propose that to assess the transactivating potential of ERα variants, the promoter focus must be expanded to include promoters that contain noncanonical regu-

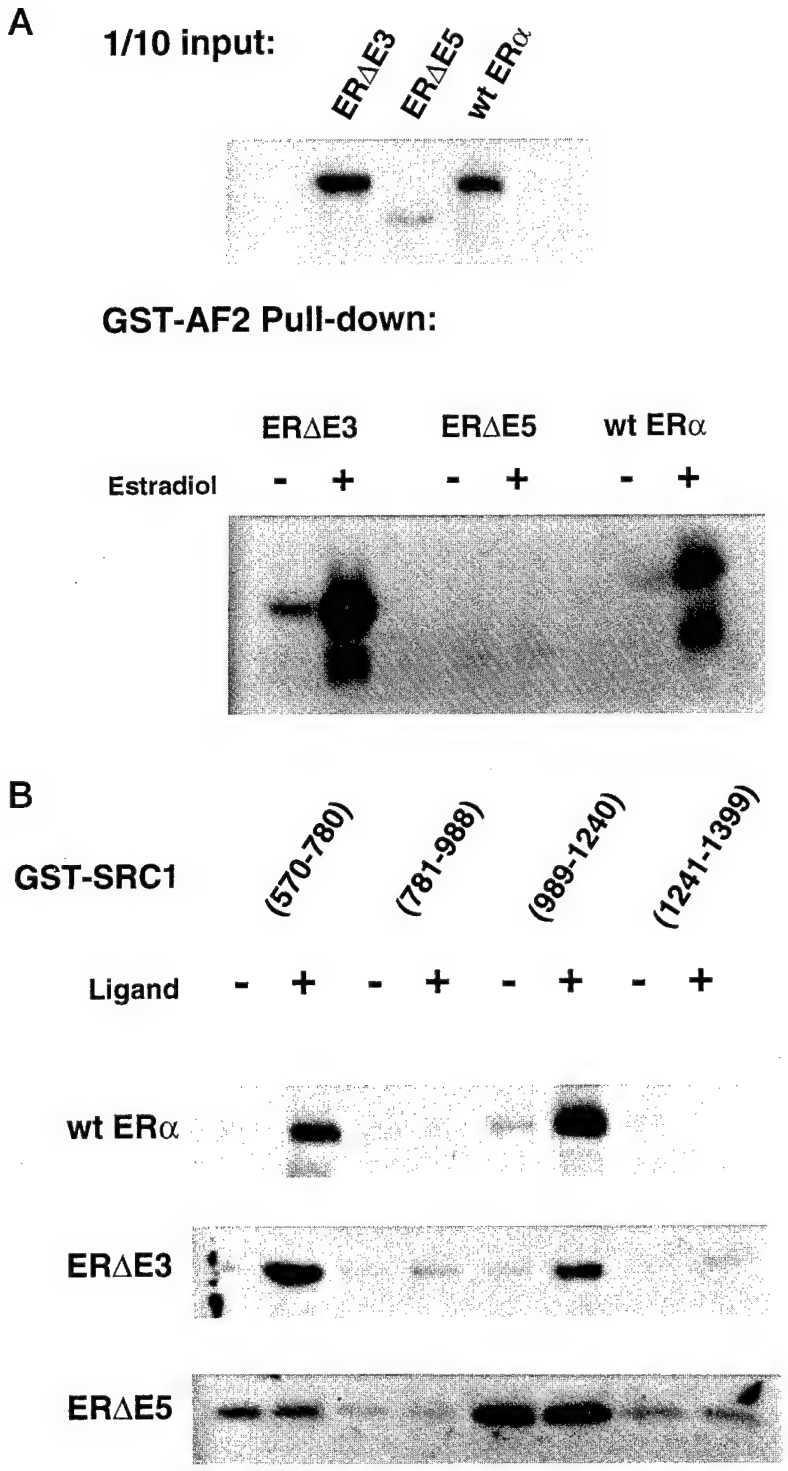

Fig. 6. *In Vitro* Binding of Radiolabeled Receptor Variants with GST-Fused wt ERα and SRC-1e Fragments

A, An autoradiograph showing *in vitro* translated, ^{35}S-methionine-labeled wt ERα and ERΔE3 retained by the AF2 domain of wt ERα receptor fused to GST (GST-AF2) and complexed to glutathione-Sepharose beads. Dimer formation requires the presence of 2.5 μM E_2. ERΔE5 does not bind to the AF2 domain of ER. *Top panel* represents 10% of the radiolabeled input. B, In the presence of ligand, wt ERα and ERΔE3 bind to SRC-1e fragments comprising amino acids 570–780 and 989–1240. The *lower panel* shows a strong constitutive interaction between radiolabeled ERΔE5 and SRC-1e fragment (989–1240).

latory elements. These thoughts prompted us to test the activity of the ERα variants on the ovalbumin promoter that contains a complex hormone response element. We performed cotransfection experiments in HeLa cells using vectors expressing wt ERα or the exon-skipped variants ERΔE2 through ERΔE7 and a CAT reporter gene construct, pOvalb-CAT, driven by a fragment of the ovalbumin promoter (−1342 to +7 relative to the transcription start site) described to encompass much of the regulatory sequence of this gene (35, 42). Results from these experiments indicate that both wt ERα and ERΔE3 support inducible gene expression from the ovalbumin promoter (Fig. 7) and that all of the remaining single exon-skipped variants are transcriptionally inactive on this reporter construct (data not shown). For wt ERα, this corroborates previously published reports (29, 35). Maximal activity was measured in cultures treated with both phorbol 12-myristate, 13-acetate (PMA, a phorbol ester) and E_2, where a 16-fold induction was observed. Like wt ERα, ERΔE3 reproducibly induced this reporter, despite its lack of an intact DBD. While the induction shown in Fig. 7 for ERΔE3 (averaging 9-fold) is less than that supported by wt ERα, the activity of ERΔE3 equaled and occasionally exceeded that of the intact receptor in several individual experiments, confirming that this variant can be a potent inducer of transcription. In both cases cotreatment with PMA and E_2 is highly synergistic as E_2 treatment alone has no significant effect, and PMA treatment alone supports only modest induction for wt ERα (2.5-fold relative to vehicle control, $P < 0.001$). Tamoxifen treatment of wt ERα- or ERΔE3-transfected cultures, either alone or together with phorbol ester, had no significant effect on pOvalb-CAT expression. This contrasts with the stimulatory activity of tamoxifen observed on other AP-1 containing estrogen-responsive reporter genes (32). In control cells transfected with an empty CMV expression vector, treatment with PMA yielded negligible reporter gene activity. This suggests that, in the absence of ERα, activation of endogenous AP-1 alone is not an effective inducer of transcription from the ovalbumin promoter in these cells. To confirm that wt ERα and ERΔE3 cooperate with AP-1 factors to regulate transactivation of the ovalbumin promoter, we measured pOvalb-CAT expression in HeLa cells cotransfected with both a receptor isoform and cJun. Transcriptional activity of wt ERα and ERΔE3 supported by PMA and E_2 cotreatment was enhanced by cJun overexpression. While the presence of endogenous AP-1 tended to obscure the synergism between cJun and wt ERα in this system, the combined effects of these transcription factors were slightly more than additive. A greater than additive activation was also

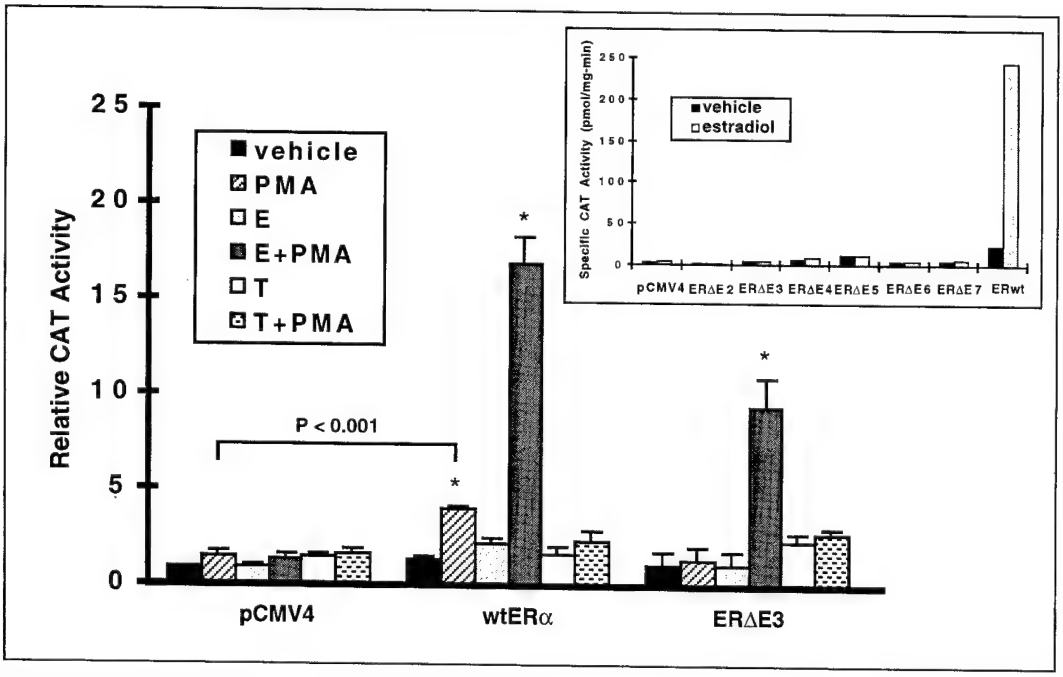

Fig. 7. Transcriptional Activity of wt ERα and Receptor Variants

Only ERΔE3 supports CAT gene expression from the ovalbumin promoter, similar to wt ERα. HeLa cells were cotransfected with 16 μg of reporter (pOvalb-CAT) and 0.5 μg of the indicated ERα expression vector followed by 24 h of hormone treatment in the presence of 5% charcoal-treated calf serum. Cultures were treated as indicated: vehicle control; 2×10^{-8} M PMA; 10^{-8} M 17β-estradiol (E); E + PMA; and 10^{-7} M tamoxifen (T) or T + PMA. CAT assays were normalized for equal amounts of protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. *Error bars* represent the SEM of three independent experiments (*, $P < 0.001$). As shown in the *inset*, aside from wt ERα itself, none of the ERα splicing variants tested are strong activators of the ERE-driven CAT reporter gene (pERE-TK-CAT) in HeLa cells.

observed when cJun and ERΔE3 were coexpressed (Fig. 8). Exogenous cJun alone elicited only a modest response to PMA and E_2 treatment. These observations, combined with the dual requirement for activating both AP-1 and ERα, strongly suggest that these factors are acting cooperatively on the ovalbumin promoter.

DISCUSSION

Our efforts to functionally characterize exon-skipped ERα mRNA splicing variants have identified two receptor isoforms that possess the ability to modulate estrogen signaling on genes that are targeted by the ER. Although their protein structure is significantly altered, the ERΔE3 and ERΔE5 splicing variants retain many of the activities attributed to the full-length receptor. Loss of exon 3 results in a receptor protein with an internal deletion that lacks a major portion of the DBD and therefore prevents ERΔE3 from binding to a consensus ERE, as confirmed by gel mobility shift analysis. However, ERΔE3 retains the LBD and NLS, thereby allowing it to bind hormone with an affinity similar to wt ERα and translocate to the nucleus. The deletion of exon 5 causes a frame-shift mutation and results in a C-terminally truncated form of the receptor. Loss of the LBD predictably renders ERΔE5 unable to bind E_2. Nonetheless, ERΔE5 still retains the NLS, and immunofluorescence analysis shows nuclear staining in Cos7 cells transfected with this variant.

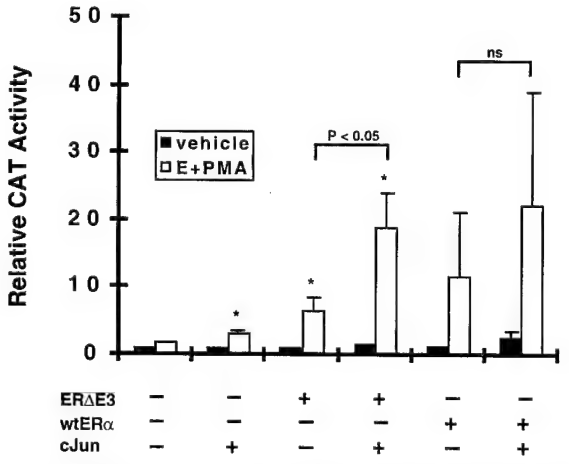

Fig. 8. Effect of cJun Overexpression on wt ERα and ERΔE3 Activation of the Ovalbumin Promoter

Cotransfection of a cJun expression vector enhances both wt ERα and ERΔE3 transactivation of the pOvalb-CAT reporter gene in HeLa cells cotreated with vehicle or 2×10^{-8} M PMA and 10^{-8} M 17β-estradiol (E + PMA). The addition of cJun increased the induced activity of the ovalbumin promoter approximately 2-fold relative to either receptor isoform alone. CAT assays were normalized for equal amounts of protein. Values are expressed relative to vehicle-treated empty expression vector, pCMV4. *Error bars* represent the SEM of at least four independent experiments (*, $P < 0.05$).

Rather than serving to stimulate transcription on a consensus ERE, results from transient transfection experiments in HeLa cells that combine either ERΔE3 or ERΔE5 with wt ERα and an ERE-driven reporter gene indicate that these isoforms actually function to inhibit transcriptional activation by wt ERα. These observations agree with our previous results and with those reported by others from similar experiments using HMT-3522S1 cells (38, 39). A 70% inhibition of transcriptional activation by E_2-liganded wt ERα on an ERE-driven CAT reporter gene was observed in HeLa cells when ERΔE3 and wt ERα expression vectors were cotransfected at a ratio of 5:1 (39). In the ER-negative cell line HMT-3522S1, coexpression of an equal amount of ERΔE5 significantly inhibited stimulation of an ERE reporter construct by wt ERα (38). Increasing the ratio of transfected variant to wt ERα demonstrates that the repression of wt ERα by ERΔE3 and ERΔE5 is dose-related and becomes nearly complete when the variants are present in sufficient excess (38, 39). This observation has physiological significance in the case of breast tumor cells that predominantly express one of these splicing variants (12, 22). Castles *et al.* (22) report that ERΔE5 is the major ER transcript in BT-20 and MDA MB 330 breast tumor cell lines. In BT-20 cells the ERΔE5 variant comprises 68% of the ER mRNA population while wt ERα measures 8%. Studies by Erenburg *et al.* (43) indicate that, while ERΔE3 tends to be underrepresented in breast tumors and tumor cell lines, it typically constitutes 50% or more of ERα mRNA in both stromal fibroblasts and epithelial cells isolated from reduction mammoplasty specimens. These authors further demonstrated that stable overexpression of ERΔE3 in MCF-7 cells to levels seen in normal mammary epithelial cells dramatically reduced the expression and estrogen inducibility of endogenous pS2 mRNA, as well as reducing their anchorage-independent growth and *in vivo* invasiveness (43).

The dominant negative character of ERΔE3 and ERΔE5 suggests that, like wt ERα, these variants are able to interact with at least one component of the ERE-directed transcription complex in a manner that disrupts positive gene regulation by wt ERα. Based on gel mobility shift assay analysis, it is unlikely that transcriptional interference by these variants involves binding to an ERE to the exclusion of wt ERα. Our DNA binding analysis indicates that ERΔE5 can bind only weakly to DNA, and only when the formation of this complex is stabilized by the addition of a bivalent antibody. The role of the antibody in this case is presumably to substitute for the missing dimerization interface and to tether receptor subunits together in a form more able to interact with DNA. DNA binding by ERΔE7 similarly requires the addition of antibody, but this binding is even less efficient than binding by ERΔE5. Interestingly, a correlation exists among the ERα variants between their ability to translocate to the

nucleus and their transcriptional inhibitory effect on wt ERα activity in mammalian cells. As of yet, no clear function has been established for the ERΔE7 variant in mammalian cells, despite an earlier report that ERΔE7 is a dominant inhibitor of wt ERα function in yeast (44). This is noteworthy since a number of quantitative studies have indicated that, as a rule, ERΔE7 represents the most abundant of the ERα splicing variants in breast tumors (summarized in Ref. 15).

We have previously reported that although ERΔE3 is unable to bind to an ERE itself, it can prevent wt ERα from binding to DNA (39). That ERΔE3 inhibits both DNA complex formation and transactivation by wt ERα suggests that the potential targets of interaction by ERΔE3 may include protein-protein contacts with wt ERα itself or interactions with nuclear receptor coactivators or other receptor-associated factors. ERα function may be disrupted when ERΔE3, which lacks the DBD but retains the hormone-inducible dimerization domain, forms mixed dimers with wt ERα that are inefficient at binding stably to DNA. We are able to show that, in the presence of E_2, ERΔE3 (but not ERΔE5) can form a stable complex with the LBD of ERα fused to GST attached to glutathione-Sepharose beads. This is consistent with a model for direct inhibition of the DNA binding activity of full-length receptor by ERΔE3. Experiments using fragments from SRC-1e fused to GST indicate that both ERΔE3 and ERΔE5 can bind a nuclear receptor coactivator. Similar to the pattern of wt ERα interaction with SRC-1e, *in vitro* translated ERΔE3 is able to associate in an E_2-dependent manner with two regions of the steroid coactivator SRC-1e (amino acids 570–780 and 989-1240). This agrees with previous reports that also describe three conserved nuclear receptor-binding motifs (LXXLL) within the 570–780 amino acid region and a distinct site for AF-1 interaction within the 989-1240 amino acid fragment (17, 45). A site for SRC-1 interaction within ERα corresponds with the AF2 domain (46), a region that is retained in the ERΔE3 variant. Isoforms of SRC-1 are potent enhancers of agonist-bound ERα and are required for its full transcriptional activity (47, 48). Transfection experiments in E_2-treated HeLa cell cultures demonstrate that coexpression of mutants containing the C terminus of ERα can attenuate ERα-dependent gene expression and that this decreased activity can be overcome with simultaneous overexpression of the SRC-1-related coactivator transcriptional intermediary factor 2 (TIF2) (49). These results suggest that coactivators are limiting factors for which the receptors are competing and that ERΔE3, like wt ERα, is a target for SRC-1 binding.

In a surprising result from cotransfection studies using engineered mutants of ERα, maximal expression of an ERE-containing reporter gene could be observed when SRC-1 was transfected simultaneously with separate N- and C-terminal fragments of ERα, containing the AF-1/DBD and the LBD/AF2 regions, respectively (50). These results suggest that separate AF1- and AF2-containing ERα polypeptides can interact in a transcriptionally productive manner, provided they are brought together by SRC-1. Furthermore, they provide an initial indication that SRC-1 interacts separately and perhaps directly with both the AF1 and AF2 domains. More support for this notion is provided by our observations that ERΔE5 binds to the SRC-1e amino acid fragment 989-1240 in solution. These results suggests the possibility that the inhibitory function of ERΔE5, which itself is relatively inefficient at binding DNA or activating transcription through an ERE, most likely results from competition with wt ERα for interaction with SRC-1 or other cellular factors.

The most compelling evidence that some of the ERα mRNA splicing variants may indeed be transcriptionally active is seen in transfection experiments involving ERΔE3 and reporter gene constructs containing a nonconsensus hormone-regulatory element. Recently, a novel mechanism for mediation of an estrogen response has been reported to involve AP-1-directed regulation of transcription by ERα (29–32). AP-1 and its isoforms represent a family of nearly ubiquitous transcription factors whose activity is crucial for the efficient expression of a wide variety of genes. As an important downstream target for the mitogen-activated protein kinase (MAPK)- and Jun kinase signaling cascades, AP-1 is a central player in mediating the effects of serum and growth factors on cellular proliferation (33, 34). A variety of estrogen-responsive genes have been described that lack a palindromic ERE, but instead contain one or more consensus AP-1 elements that often occur near a degenerate ERE or ERE half-site (29, 31, 32). It should be noted that these imperfect EREs may in some cases serve dual function as cryptic AP-1 response elements whose consensus sequence (5′-TGAG/CTCA-3′) bears superficial similarity to an ERE half-site (5′-GGTCA-3′ or 5′-TGACC-3′).

An important observation from the analysis of genes regulated by noncanonical EREs is that the structure-activity requirements for activation by ERα (both for the ligand and for the receptor) are different than those for transcriptional activation through a palindromic ERE. Using a region of the collagenase gene promoter (−73/+63) that lacks an ERE but harbors an essential AP-1 element, Kushner and co-workers (32) demonstrated that a DBD-deleted mutant of ERα was significantly more effective at supporting E_2-induced reporter gene expression than wt ERα in transfected HeLa cells. Similar to collagenase gene expression, ERα-dependent activation of the chicken ovalbumin promoter, which lacks a palindromic ERE, does not require an intact DBD (29). Furthermore, an ERE half-site was determined to be the site for synergistic regulation of ovalbumin gene expression by AP-1 and ERα (29). Our studies involving cotransfection of an ERα splice variant with the ovalbumin promoter construct, pOvalb-CAT, agree with these findings. Results from Fig. 7 demonstrate that, compared with mock-transfected HeLa control cultures, pOvalb-CAT is strongly activated by either wt ERα or ERΔE3. A breakdown of treatments indicates that maximal activity of

both wt ERα and ERΔE3 clearly requires E_2 in addition to an AP-1 activator. *In vitro* assays demonstrate an interaction between cJun and the N terminus of ERα fused to GST (32). Additional evidence suggesting that ERΔE3 and wt ERα cooperate with activated AP-1 to maximally transactivate the ovalbumin promoter is provided by our observation that receptor activity is enhanced by simultaneous cJun overexpression. In our studies tamoxifen treatment had little or no effect on the activity of wt ERα or ERΔE3, either with or without PMA cotreatment. This contrasts with results observed when wt ERα was cotransfected with a collagenase reporter construct in HeLa cells, where tamoxifen supported a significant induction of reporter gene expression (32).

Our transfection results show that, while several of the ERα splicing variants are functionally incapacitated by their deletions, two of the variants clearly retain significant transcriptional activity. For the ERΔE3 and ERΔE5 variants, this activity is quite complex. Both of these variants represent stable receptor isoforms that, like the full-length receptor, localize efficiently to the nucleus where they can interact with the transcription apparatus. However, when acting through a consensus ERE, these variants completely lack (ERΔE3) or show only weak (ERΔE5) transcriptional stimulatory activity, consistent with their poor DNA binding ability. On the contrary, both variants serve to blunt the ability of coexpressed wt ERα to promote transcription of ERE-containing genes. At the same time, the ability of ERΔE3 (and presumably also ERΔE5) to interact productively with nuclear receptor coactivators or other transcription factors gives these ERα splicing variants the potential to stimulate or otherwise modulate gene expression through nonconsensus hormone response elements that are targeted by AP-1 motifs or other DNA-binding sites. We have clearly shown this to be true for ERΔE3 and the chicken ovalbumin promoter and believe that this is also likely to be true for many other genes, such as those encoding collagenase, cathepsin D, IGF-I, transforming growth factor-β, c-fos, heat shock protein-27, and retinoic acid receptor-α, all of which lack an obvious ERE and yet still respond to estrogen. In this respect, ERα splicing variants may actually serve to redirect transcription away from ERE-containing genes to genes such as these that appear to be regulated nonclassically by estrogens.

MATERIALS AND METHODS

Expression Vectors

Plasmids for ERα mRNA splicing variant cDNAs were generated as derivatives of pCMV4 (51) and pcDNA3.1 (Invitrogen, San Diego, CA), which support high levels of receptor expression in HeLa and Cos7 cell lines (41). Plasmids expressing ERΔE4, ERΔE5, and ERΔE6 were generated using synthetic oligonucleotides to construct the variant splice junctions within an otherwise wt ERα cDNA expression plasmid. The remaining plasmids were constructed with the use of flanking restriction sites to shuttle cloned cDNAs (39) into the appropriate expression vectors. Mouse cJun cDNA cloned into the pCMV2 expression vector was provided by L. McCabe (Michigan State University, East Lansing, MI).

Cell Culture, Transfection, and CAT Assays

Cos7 and Hela cells were grown in phenol red-free DMEM supplemented with 10% calf-serum, 5 mM HEPES (pH 7.4), 2 mM glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Cells were transfected by the $CaPO_4$ method, as previously described (52). HeLa cells ($\sim 2 \times 10^{-6}$ cells per 100-mm dish) were transfected with 1 μg of the indicated ERα expression plasmid, 2 μg of the cJun expression plasmid (where indicated) and 16 μg of the estrogen-responsive reporter plasmid, pERE-TK-CAT (53) or pOvalb-CAT (a reporter gene construct containing −1342 to +7 bp of the chicken ovalbumin promoter relative to its transcription start site) (42). Calf thymus DNA (10 μg) was added as carrier. After overnight incubation with DNA, culture medium was replaced with 5% charcoal-treated serum-supplemented DMEM containing the indicated hormones. After a 24-h incubation, cells were harvested and CAT assays were performed as previously described (54) using 100 μg protein. Quantification of CAT activities was performed by phosphorimage analysis of thin layer chromatographs (ImageQuaNT, Molecular Dynamics, Inc., Sunnyvale, CA). For experiments involving biochemical or cytochemical analysis of ERα variants, Cos7 cells were similarly transfected with 10 μg of the indicated expression plasmid and 10 μg of calf thymus carrier DNA. After overnight exposure to DNA, cells were cultured for 48 h in 10% calf serum-supplemented DMEM. All experiments involving extracts from transfected cells were normalized with respect to protein, as measured using the method of Lowry *et al.* (55). Two-way ANOVA and comparison with Student's *t* test were used to assess statistical differences between groups. Statistical significance was set at the $P < 0.05$ or $P < 0.001$ level as indicated in Figs. 7 and 8.

E_2 Binding Analysis

Ligand-binding assays were performed as previously described (40). Whole-cell extracts were prepared from transfected Cos7 cells that were resuspended and sonicated in extraction buffer (20 mM HEPES, pH 7.4, 20% glycerol, 0.4 M KCl, 1 mM $MgCl_2$) supplemented immediately before use with protease inhibitors (0.05 mg/ml each of chymostatin, trypsin inhibitor, antipain, leupeptin, aprotinin, and pepstatin). Aliquots containing 200 μg of protein were incubated overnight at 4 C with various concentrations (0.1 nM–10 nM) of ^3H-labeled E_2 (NEN Life Science Products, Boston, MA) in the presence or absence of a 200-fold molar excess of unlabeled E_2. Free ligand was separated from bound ligand by treatment with dextran-coated charcoal. For determination of equilibrium binding constants, these data were plotted according to the method of Scatchard (56).

DNA Binding Assays

DNA binding assays were performed as previously described (40). Aliquots containing 30 μg of protein from extracts prepared as above from transfected Cos7 cells were preincubated for 15 min at room temperature in 10 μl binding buffer [10 mM HEPES (pH 7.4), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 20% glycerol] containing 1 μg poly (dI-dC), with or without 1 μl of added human ER-specific monoclonal antibody (Mab-17), generated as described by Neff *et al.* (40). Approximately 6 fmol (40,000 cpm) of a ^{32}P-labeled double-stranded ERE oligonucleotide (39) were added to the samples and incubated for 30 min at room temperature, followed by an addi-

tional 5-min incubation at 4 C. Samples were then loaded on a preelectrophoresed nondenaturing 5% polyacrylamide gel that was run in 0.5 × Tris-Borate-EDTA at 275 V for 2 h. The gel was dried and exposed for autoradiography.

Immunoblot Analysis

Discontinuous 12% SDS-PAGE was carried out as previously described (57). After electrophoresis of 30 μg of whole-cell protein from extracts of transfected Cos7 cells, proteins were electrophoretically transferred to nitrocellulose filters with a Trans Blot apparatus (Bio-Rad Laboratories, Inc. Richmond, CA) using the procedure of Erickson *et al.* (58). Immunoblots were probed with the ER-specific monoclonal antibody, Mab-17, obtained from a hybridoma culture supernate that was diluted with an equal volume of PBS (40). Immunoreactive protein was visualized by enhanced chemiluminescence using a horseradish peroxidase-conjugated goat antimouse IgG, following manufacturer's instructions (Amersham Pharmacia Biotech, Arlington Heights, IL).

In Vitro Protein-Protein Interaction Assays

Variant and wt ERα receptor protein was translated in the presence of [^{35}S]methionine using the TNT Coupled Reticulocyte System (Promega Corp., Madison, WI). GST-fusion proteins were expressed in the pGEX system (Pharmacia Biotech, Uppsala, Sweden) (45, 59). Overnight cultures of transformed bacteria were diluted 1:20 and cultured for 2 h before protein expression was induced with the addition of isopropyl β-D-thiogalactoside (IPTG, 0.2 mM final concentration). Bacteria were collected by centrifugation 2 h following IPTG induction, and pellets were resuspended in 400 μl of extraction buffer supplemented with protease inhibitors. Cells were sonicated briefly, and the resulting lysates were centrifuged for 20 min at 20,000 rpm, 4 C. Protein concentrations were determined (55) and extracts were diluted to 2 μg/μl extraction buffer and stored at −70 C until binding assays were performed.

Before use in protein interaction assays, 25 μl of glutathione-Sepharose 4B beads (Pharmacia Biotech) were washed three times in 100 μl NETN [0.5% Nonidet P-40, 1 mM EDTA, 20 mM Tris (pH 8.0) 100 mM NaCl] and suspended in 100 μl NETN, 0.5% powdered milk. Washed beads were incubated with 40 μg of GST-fusion protein for 2 h, rotating at room temperature. Beads complexed with GST-fusion proteins were washed three times with 100 μl NETN, 4 C. For protein-protein interaction assays, 5 μl of *in vitro* translated receptor were added to washed complexed beads resuspended in 100 μl NETN supplemented with protease inhibitors (as above) with and without 2.5 μM E_2. After a 2-h incubation during which the samples were rotated at room temperature, the beads were pelleted and washed four times with 100 μl NETN, 4 C. Bound proteins were separated on a discontinuous 10% polyacrylamide SDS-PAGE gel (57). The gels were dried and exposed for autoradiography.

Immunohistochemical and Cytochemical Analysis

Indirect immunofluorescence analysis was performed as previously described (40) using Cos7 cells that were plated and transfected on glass cover slips. On the second day after transfection, cells were washed three times with Tris-buffered saline (TBS), fixed for 3 min in cold 95% methanol, rehydrated by three washes with TBS, and incubated 30 min at 37 C with primary antibody (Mab-17 hybridoma supernate used at a 1:10 dilution in TBS). Bound antibody was detected by staining with a rhodamine-conjugated affinity-purified goat antimouse IgG (Roche Molecular Biochemicals, Indianapolis, IN) diluted 1:2000 in TBS, and incubating for 30 min at 37 C in the presence of 0.02 μg/ml of 4′,6-diamidine-2-phenylindole dihydrochloride. Confocal images were recorded using the Odyssey system (Noran Instruments, Middleton, WI) on an Optiphot 2 Nikon (Melville, NY) microscope. Fluorescent ligand staining of transfected Cos7 cells was performed as described by Miksicek *et al.* (41) on live, whole-cell mounts treated in DMEM with 10^{-7} M nitrile THC. For these studies, cells were visualized using a Nikon UFX microscope equipped with a 100 watt mercury lamp for fluorescence excitation, and a 40 × 0.7 numerical aperture Plan objective.

Acknowledgments

We are grateful to Malcolm G. Parker for kindly providing us with the GST-fusion constructs, to M. Sanders for pOvalb-CAT, and to L. McCabe for pCMV2-cJun. We would also like to thank M. Morrison and D. Ankrapp for helpful suggestions during the course of this work.

Received February 15, 1999. Re-revision received January 31, 2000. Accepted February 3, 2000.

Address requests for reprints to: Richard J. Miksicek, Department of Physiology, 108 Giltner Hall, Michigan State University, East Lansing, Michigan 48824-1101. E-mail: miksicek@msu.edu.

This work was supported by Awards DAMD1794-J-4372 and DAMD1799-1-9293 to R.J.M. and A.B. from the US Army Breast Cancer Research Program.

REFERENCES

1. Beato M, Sanchez-Pacheco A 1996 Interaction of steroid hormone receptors with the transcription initiation complex. Endocr Rev 17:587–609
2. Kumar V, Chambon P 1988 The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145–156
3. Tsai M-J, O'Malley BW 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63:451–486
4. Dickson Rb, Johnson MD, el-Ashry D, Shi YE, Ban O, Zugmaier G, Ziff B, Lippman ME, Chysogelos S 1993 Breast cancer: influence of endocrine hormones, growth factors and genetic alterations. Adv Exp Med Biol 330: 119–141
5. Haslam SZ 1987 Role of sex steroid hormones in normal mammary gland function. In: Neville MC, Daniel CW (eds) The Mammary Gland. Plenum, New York, pp 499–533
6. Kaufmann M 1997 A review of endocrine options for the treatment of advanced breast cancer. Oncology 54[Suppl 2]:2–5
7. Clarke M, Collins R et al. 1998 Tamoxifen for early breast cancer: an overview of randomised trials. Lancet 351: 1451–1467
8. Smigel K 1998 Breast cancer prevention trial shows major benefit, some risk. J Natl Cancer Inst 90:647–648
9. Huston L 1999 Raloxifene reduces breast cancer risk. Lancet 353:44
10. Kuipper GGJM, Enmark E, PeltoHuikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
11. Mosselman S, Polman J, Dijkema R 1996 ERβ: identification and characterization of a novel human estrogen receptor. FEBS Lett 392:49–53
12. Castles CG, Klotz DM, Fuqua SAW, Hill SM 1995 Coexpression of wild-type and variant oestrogen receptor mRNAs in a panel of human breast cancer cell lines. Br J Cancer 71:974–980

13. Gotteland M, Desauty G, Delarue JC, Liu L, May E 1995 Human estrogen receptor messenger RNA variants in both normal and tumor breast tissues. Mol Cell Endocrinol 112:1–13
14. Miksicek RJ, Lei Y, Wang Y 1993 Exon skipping gives rise to alternatively spliced forms of the estrogen receptor in breast tumor cells. Breast Cancer Res Treat 26: 163–179
15. Murphy LC, Leygue E, Dotzlaw H, Douglas D, Coutts A, Watson PH 1997 Oestrogen receptor variants and mutations in human breast cancer. Ann Med 29:221–234
16. Metzger D, Ali S, Bonnert JM, Chambon P 1995 Characterization of the amino-terminal transcriptional activation function of the human estrogen receptor in animal and yeast cells. J Biol Chem 270:9535–9542
17. Webb P, Nguyen P, Shinsako J, Anderson C, Feng W, Nguyen MP, Chen D, Katzenellenbogen BS, Stallcup MR, Kushner PJ 1998 Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol 12:1605–1618
18. Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P 1987 Functional domains of the human estrogen receptor. Cell 51:941–951
19. Ylikomi T, Bocquel MT, Berry M, Chambon P 1992 Cooperation of protosignals for nuclear accumulation of estrogen and progesterone receptors. EMBO J 11: 3681–3694
20. Kumar V, Guren S, Staub A, Chambon P 1986 Localization of the oestradiol-binding and putative DNA binding domain of the human oestrogen receptor. EMBO J 5:2231–2236
21. Horowitz KB, Jackson TA, Bain DL, Richer JK, Takimoto GS, Tung L 1996 Nuclear receptor coactivators and corepressors. Mol Endocrinol 10:1167–1177
22. Castles CG, Fuqua SAW, Klotz DM, Hill SM 1993 Expression of a constitutively active estrogen receptor variant in the estrogen receptor-negative BT-20 human breast cancer cell line. Cancer Res 53:5934–5939
23. Fuqua SAW, Fitzgerald SD, Chamness GC, Tandon AK, McDonnell DP, Nawaz Z, O'Mally BW, McGuire WL 1991 Variant human breast tumor estrogen receptor with constitutive transcriptional activity. Cancer Res 51:105–109
24. Fuqua SAW, Wolf DM 1995 Molecular aspects of estrogen receptor variants in breast cancer. Breast Cancer Res Treat 35:233–241
25. Chaidarun SS, Alexander JM 1998 A tumor-specific truncated estrogen receptor splice variant enhances estrogen-stimulated gene expression. Mol Endocrinol 12: 1355–1366
26. Wiseman LR, Johnson MD, Wakeling AE, Lykkesfeldt AE, May FEB, Westley BR 1993 Type I IGF receptor and acquired tamoxifen resistance in oestrogen-responsive human breast cancer cells. Eur J Cancer 29A:2256–2264
27. Wolf DM, Jordan VC 1994 Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice. Breast Cancer Res Treat 31:117–127
28. Rea D, Parker MG 1996 Effects of an exon 5 variant of the estrogen receptor in MCF-7 breast cancer cells. Cancer Res 56:1556–1563
29. Gaub MP, Bellard M, Scheuer I, Chambon P, Sassone-Corsi P 1990 Activation of the ovalbumin gene by the estrogen receptor involves the Fos-Jun complex. Cell 63:1267–1276
30. Uht RM, Anderson CM, Webb P, Kushner PJ 1997 Transcriptional activities of estrogen and glucocorticoid receptors are functionally integrated at the AP-1 response element. Endocrinology 138:2900–2908
31. Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y, Kamada T 1994 Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. J Biol Chem 269:16433–16442
32. Webb P, Lopez GN, Uht RM, Kushner PJ 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9:443–456
33. Boyle WJ, Smeal T, Defize LHK, Angel P, Woodgett JR, Karin M, Hunter T 1991 Activation of protein kinase C decreases phosphorylation of cJun at sites that negatively regulate its DNA-binding activity. Cell 64: 573–584
34. Westwick JK, Weitzel C, Minden A, Karin M, Brenner DA 1994 Tumor necrosis factor a simulates AP-1 activity through prolonged activation of the c-Jun kinase. J Biol Chem 269:2639–26401
35. Tora L, Gaub MP, Mader S, Dierich A, Bellard M, Chambon P 1988 Cell-specific activity of a GGTCA half-palindromic oestrogen-responsive element in the chicken ovalbumin gene promoter. EMBO J 7:3771–3778
36. Ernst M, Rodan GA 1991 Estradiol regulation of insulin-like growth factor-1 expression in osteoblastic cells: evidence for transcriptional control. Mol Endocrinol 5:1081–1089
37. Murphy LJ, Ghahary A 1990 Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. Endocr Rev 3:443–453
38. Ohlsson H, Lykkesfeldt AE, Madsen MW, Briand P 1998 The estrogen receptor variant lacking exon 5 has dominant negative activity in the human breast epithelial cell line HMT-3522S1. Cancer Res 58:4264–4268
39. Wang Y, Miksicek RJ 1991 Identification of a dominant negative form of the human estrogen receptor. Mol Endocrinol 5:1707–1715
40. Neff S, Sadowski C, Miksicek RJ 1994 Mutational analysis of cysteine residues within the hormone-binding domain of the human estrogen receptor identifies mutants that are defective in both DNA-binding and subcellular distribution. Mol Endocrinol 8:1215–1223
41. Miksicek RJ, Carlson KE, Hwang K-J, Katzenellenbogen JA 1995 Studies using fluorescent tetrahydrochrysene estrogens for *in situ* visualization of the estrogen receptor in living cells. Mol Endocrinol 9:592–604
42. Schweers LA, Frank DE, Weigel NL, Sanders MM 1990 The steroid-dependent regulatory element in the ovalbumin gene does not function as a typical steroid-response element. J Biol Chem 265:7590–7595
43. Erenburg I, Schachter B, Mira y Lopez R, Ossowski I 1997 Loss of an estrogen receptor isoform (ER$\alpha\Delta$E3) in breast cancer and the consequences of its reexpression: interference with estrogen-stimulated properties of malignant transformation. Mol Endocrinol 11:2004–2015
44. Fuqua SAW, Fitzgerald SD, Allred DC, Elledge RM, Nawaz Z, McDonnell DP, O'Malley BW, Green GL, McGuire WL 1992 Inhibition of estrogen receptor action by a naturally occurring variant in human breast tumors. Cancer Res 52:483–486
45. Kalkhoven E, Valentine JE, Heery DM, Parker MG 1998 Isoforms of steroid receptor co-activator 1 differ in their ability to potentiate transcription by the oestrogen receptor. EMBO J 17:232–243
46. Feng W, Ribeiro RCJ, Wagner RL, Nguyen H, Apriletti JW, Fletterick RJ, Baxter JD, Kushner PJ, West BL 1998 Hormone-dependent coactivator binding to a hydrophobic cleft on nuclear receptors. Science 280:1747–1749
47. Oñate SA, Tsai SY, Tsai M-J, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. Science 270: 1354–1357
48. Xu J, Qiu Y, DeMayo FJ, Tsai SY, Tsai M-J, O'Malley BW 1998 Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 279:1922–1925
49. Vogel JJ, Heine MJS, Zechel C, Chambon P, Gronemeyer H 1996 TIF2, a 160 kDa transcriptional mediator

for the ligand-dependent activation function AF-2 of nuclear receptors. EMBO J 15:3667–3675
50. McInery EM, Tsai MJ, O'Malley BW, Katzenellenbogen BS 1996 Analysis of estrogen receptor transcriptional enhancement by a nuclear hormone receptor coactivator. Proc Natl Acad Sci USA 93:10069–10073
51. Andersson S, Davis DN, Dahlback H, Jornvall H, Russell DW 1989 Cloning, structure, and expression of the mitochondrial cytochrome P450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 264:8222–8229
52. Jordan M, Schallhorn A, Wurm FM 1996 Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. Nucleic Acids Res 24:596–601
53. Klock G, Strähle U, Schütz G 1987 Oestrogen and glucocorticoid responsive elements are closely related but distinct. Nature 329:734–736
54. Gorman CM, Moffat LF, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044–1051
55. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
56. Scatchard G 1949 The attractions of proteins for small molecules and ions. Ann NY Acad Sci 51:660–672
57. Laemmli UK 1970 Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685
58. Erickson PF, Minier LN, Lasher RS 1982 Quantitative electrophoretic transfer of polypeptides from SDS polyacrylamide gels to nitrocellulose sheets: a method for their re-use in immunoautoradiographic detection of antigens. J Immunol Methods 51:241–249
59. Cavailles V, Dauvois S, Danielian PS, Parker MG 1994 Interaction of proteins with transcriptionally active estrogen receptors. Proc Natl Acad Sci USA 93:10009–10013